Figure 1 illustrates the effects of GMCHA derivatives on cell growth, DNA synthesis and proteinase In activity in synchronized *E. coli* Cells. *Left*, Growth of synchronized cells in the absence of compound PH04. *(a)*, Viable cell number and [^{3}H] thymidine uptake were determined at 5-min intervals, and proteinase In activity was determined at 30-sec intervals. Time zero was set at the first sign of cell division. *(b)* and *(c)*, Appearance of proteinase In activity at -30 min and +30 min, respectively. P, Q, and R represent the cell division period, the period between cell division and initiation of chromosome replication, and the period between initiation of chromosome replication and cell division, respectively. *Right*, Growth of synchronized cells in the presence of 27μM compound PH04. *(a)*, The inhibitor was added at -8 min (arrow). The sample was as described above. Note that without PH04, duration of P, Q, R1, and R2 was 15, 15, 15, and 10 min, respectively, whereas with PH04, DNA synthesis was initiated at 30min and DNA synthetic activity doubled at 65min. The R1 period was prolonged from 15 to 35 min. *(b)*, Appearance of proteinase activity at about 30 min as in control cells, but had a prolonged half-life.

Since the GMCHA derivatives were originally identified as synthetic trypsin inhibitors in vitro, we investigated whether a trypsin-like protease could be detected in *E. coli* extracts using as fluorogenic substrate Boc-Val-Pro-Arg-NH-Mec. (Kato et al., *Eur. J. Biochem.*, 210:1007-1014 (1992)). We detected a single activity and had purified it to homogeneity. This was achieved in eight steps, with a 2,880-fold purification and a yield of 15 percent. The purified enzyme has a molecular mass of ~66 kDa and an isoelectric point of 4.9. Based upon pH optimum, hydrolytic activity on various synthetic substrates, and effects of various known proteolytic inhibitors, this *E. coli* protease has very different specificity than mammalian trypsin or any of known bacterial enzymes. Most exciting is the finding that the sensitivity of the purified enzyme to various GMCHA derivatives paralleled exactly their inhibitory effects on *E. coli* (Table 1). This latter finding may suggest that this protease is the molecular target within the *E. coli* cell for this class of growth inhibitory compounds. We had gone on to demonstrate, using synchronized *E. coli* cultures, that expression of this protease is restricted to just before the initiation of chromosomal DNA replication (Fig. 2, left). (Kato et al., *Biol.Pharm. Bull.*, 16:552-557 (1993)). Furthermore, addition of the 4-tert-butylphenyl (PH04) derivative after DNA synthesis had initiated did not affect that round of cell division but

retarded the next cycle. However, addition of the inhibitor prior to the initiation of DNA replication resulted in prolongation of that same round of cell division (Fig. 1, right). Together, these results suggest that this protease probably participates directly in the initiation of chromosome replication in *E. coli*, and that it is the target of inhibition by the aromatic esters of GMCHA. We named this enzyme proteinase In, and its gene was identical with *prlC*. The *prlC* gene encoded a 67kDa protein with two active sites for proteinase In and oligopeptidase A. (Jiang et al., *J. Biochem.*, 124:980-985 (1998)). A proteinase In like proteinase was partially purified from *B. Subtilis* and it was stonily inhibited by various esters of GMCHA, and their effects were co-related with their inhibitory effects on the growth of the bacteria. (Irisawa et al., *Biol. Pharm. Bull.*, 16:1211-1215 (1993)). These results strongly suggest the ubiquitous occurrence of proteinase In or proteinase In-like proteinase in various bacteria, and a strong inhibitor for the proteinase is useful as an new type of antibacterial agent.

Example 3. Activities of NE-2001

A novel compound reported here 4-(4-methylbenzyl)-4'-[guanidinomethylbenzoyloxy] biphenyl-4-carboxylate, NE-2001, specifically inhibited the growth of *H. pylori* and completely eradicated *H. pylori* at various pH.

MICs (μg/ml) of several substances against 9 strains (ATCC43504, ATCC43629, ATCC43526,ATCC43579,ATCC49503,ATCC51110,ATCC51652,ATCC51653, ATCC51932) of *H. pylori* were examined. NE-2001 had minimal inhibitory concentrations (MICs) ranging from 0.10 to 0. 48 μg/ml. Summary is shown in the following Table 3.

Table 3. Summary of MIC Range against H. pylori

Substance	MIC Range (μg/ml)
NE-2001	0.10-0.48
Amoxicillin	0.01-0.08
Clarithromycin	0.01-0.90
Metronidazole	0.65-2.45

Figure 2 illustrates the anti-*H. pylori* effects of the compound NE-2001. Bactericidal effect was examined at various concentration (0.15-2.50μg/ml) for 168 hours. The representative curve was shown in Fig 2. NE-2001 showed strong bactericidal effect against *H. pylori*. No visible organism was detected in 3hr at a concentration of ≥ 1.25μg/ml. NE-2001 showed bactericidal effect against *H. pylori* in all tested pH range (pH3-7) (See Figure 3).

Figure 3. Illustrates the anti-*H.pylori* effects of the compound NE-2001 at various pH values. The appearance frequency of natural resistant mutations against NE-2001 was examined (Table 4). Obviously no natural resistant bacteria appeared at all tested concentration of NE-2001 (0.30-1.20μg/ml).

Table 4. Appearance Frequency of Natural Resistant Mutation against NE-2001

Organism	Selected Concentration Of NE-2001 (μg/ml)	Frequency
H.pylori ATCC43504	0.30 0.60 1.20	$< 3.4\times10^{-8}$ $< 3.4\times10^{-8}$ $< 3.4\times10^{-8}$

A study on a single dose toxicity of NE-2001 was performed orally in male mice. At a dose of 2000mg/kg of NE-2001 (maximum dose prepared as 0.5% methylcellulose suspension), no animal deaths occurred and all animals gained weight. General toxicity was also observed in neither cases. Apparently, NE-2001 is a safe (Table 5).

Table 5. NE-2001 Single Dose Toxicity in Mice

Animal /Age	Mouse/6 Weeks
Administration Route	Oral
Sex	Male
No. Of Animals/ Group	5
LD_{50} (mg/kg)	>2000